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Short communication

Mixed *Giardia duodenalis* assemblage A, B, C and E infections in pet chinchillas (*Chinchilla lanigera*) in Flanders (Belgium)

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ABSTRACT

Worldwide there is an increasing trend to keep exotic animals as pets. In contrast to domestic animals, few studies have addressed the importance of infectious diseases of these exotic animals harbor. Chinchillas are host to *Giardia*, but prevalence studies are scarce. Moreover, little is known about their role as a zoonotic reservoir for *Giardia*. Therefore, the objective of the present study was (1) to study the occurrence of *Giardia* in pet chinchillas, (2) to identify risk factors and (3) to determine the role of these animals as potential zoonotic reservoir. To this end, pet chinchillas (*Chinchilla lanigera*) from both pet owners and breeders in Flanders (Belgium) were screened for the presence of *Giardia* spp. using a sedimentation flotation technique. A questionnaire was distributed among the pet owners to identify putative risk factors. A subset of the *Giardia*isolates was characterized by direct sequencing of the β-giardin gene. In addition, assemblage specific PCRs targeting the triose phosphate isomerase (tpi) gene were performed. Of the 80 chinchillas screened, 53 (66.3%) excreted cysts of *Giardia*. Youngsters and animals participating in shows were significantly more at risk for infection. A total of 22 isolates were characterized. Direct sequencing of the β-giardin gene solely revealed the presence of *G. duodenalis* assemblage B. The assemblage specific PCRs confirmed these findings, but also revealed the presence of assemblage A (11 samples), C (15 samples) and E (2 samples). This study indicates that multiple *Giardia* spp. are highly prevalent in pet chinchillas and that these animals are a potential reservoir for zoonotic transmission. In addition, the results highlight the benefit of using an assemblage specific PCR in molecular studies as mixed infections are likely to be missed using conventional PCR approaches.

1. Introduction

Giardia duodenalis is a flagellate that infects a wide range of vertebrate hosts, and is a significant cause of diarrhoea and failure to thrive, particularly in young subjects. Molecular analysis of *G. duodenalis* isolates from the different host species has revealed the presence of eight different assemblages (A–H) (Lasek-Nesselquist et al., 2010), of which only assemblages A and B are of zoonotic importance. Other assemblages have a more distinct host range (Xiao and Fayer, 2008; Sprong et al., 2009). Chinchillas are rodents native to Latin America, but are currently found worldwide both as commercial (fur) and companion animals. Although *G. duodenalis* has been found in stool of chinchillas (Gurgel et al., 2005; Fialho et al., 2008), little is known about the epidemiology of *G. duodenalis* in these animals, including prevalence and factors contributing to infections. In addition, the zoonotic assemblage A was detected in chinchillas

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(Karanis and Ey, 1998). However, the distribution of other assemblages within chinchillas remains unclear. Moreover, a mixed infection with different assemblages could not be ruled out, as the standard PCR approach will preferentially amplify the most abundant assemblage (Weiss et al., 1992; Geurden et al., 2007).

Therefore, the objective of the present study was (1) to study the occurrence of *Giardia* in pet chinchillas, (2) to identify risk factors and (3) to determine the role of these animals as a potential zoonotic reservoir.

2. Materials and methods

2.1. Animals and sampling

A total of 80 animals from 4 pet owners (34) and 4 breeders (breed animals for commercial purposes) (46) in Flanders (Belgium) were included in the present study. They were housed in 34 enclosures in groups with a median of two animals (25th percentile = 2 and 75th percentile = 3). The sex ratio (female/male) of the population was 1.5 and the median age of the animals was 18 months (25th percentile = 8 and 75th percentile = 36). Fresh fecal samples were collected from individual animals on a single occasion between October–December 2007 and were processed within 24 h. None of these animals showed clinical symptoms of gastro-enteritis at the time of sampling.

2.2. Coprological examination

All samples were examined microscopically for the presence of *Giardia* spp. using a gradient centrifugation-flotation technique with sucrose with a specific density of 1.27 (Claerebout et al., 2009). Due to small quantities (<2 g), the entire fecal sample was processed to increase the sensitivity. None of the samples were pooled.

2.3. Molecular characterization

For each cage, one random sample containing *Giardia* cysts was withheld for further molecular analysis. DNA was extracted using the QIAamp® Stool Mini Kit (Qiagen) according to the manufacturer's instructions, incorporating an initial step of three freeze–thaw cycles (freezing in liquid nitrogen for 5 min and heating at 95 °C for 5 min) in the protocol to maximise rupture of the cysts. The final molecular identification was based on two approaches: direct sequencing and assemblage-specific amplification.

For the direct sequencing, the β-giardin gene was targeted as previously described (Cacciò et al., 2002; Lalle et al., 2005). The PCR products were purified using the QIAquick® PCR purification kit (QIAGEN) and fully sequenced using the BigDye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were analyzed on a 3100 Genetic Analyzer (Applied Biosystems) and assembled with Seqman II (DNASTAR, Madison, WI, USA). Subsequently, the fragments were aligned using MegAlign (DNASTAR, Madison, WI, USA) and compared with reference sequences summarised in recent studies (Cacciò et al., 2008; Wielinga and Thompson, 2007).

The assemblage-specific amplification consisted of a nested PCR targeting the tpi gene. The first reaction was based on the internal set of primers described by Sulaiman et al. (2003), followed by separate assemblage-specific nested PCRs for the detection of assemblage A–E. The amplification of *G. duodenalis* assemblage A and E was according to Geurden et al. (2007); for assemblage B the protocol described by Levecke et al. (2009) was used. For the detection of assemblage C new primers (AssemCF: 5′-GAC TCT GTT GAT GTG ATC ATC-3′ and AssemCR: 5′-AGA CGA TGA CGA CAC TCT TC-3′) were designed based on Genbank accession nos. L02120 (assemblage AI), U57897 (assemblage AII), DQ650648 (assemblage AIII), AY368163 (assemblage BIII), AY368166 (assemblage BIII-like), AY368170 (assemblage BIV), AY228635 (assemblage BIV-like), AF069563 (assemblage C), DQ246216 (assemblage D), AY655705 (assemblage E), AF069558 (assemblage F) and AF069562 (assemblage G). The GoTaq® Flexi DNA Polymerase kit (Promega) was used for each of the PCR mixtures and consisted of 2.5 μl DNA, 0.5 μl of each primer (10 μM), 1 μl $MgCl_2$ (25 mM), 5 μl GoTaq® Flexi Buffer, 14.875 μl PCR H_2O and 0.125 μl GoTaq® Flexi DNA polymerase. Identical conditions were used for both assemblage-specific PCRs: 35 cycles (95 °C for 45 s, 62 °C for 45 s and 72 °C for 1 min), with an initial step of 2 min at 95 °C and a final extension step at 72 °C for 5 min. All PCR products were electrophoresed on 1.5% agarose gels and visualised with ethidium bromide. In each PCR run a negative control (PCR H_20) and positive control for assemblage A (nonhuman primate), B (nonhuman primate), C (dog), D (dog) and E (calf) were included. To determine the subassemblages and to evaluate the specificity of the assemblage-specific primers, a subset of the samples were sequenced. To this end, the obtained PCR products were purified with QIAquick purification columns (QIAGEN, Germany) and were sequenced using the Big Dye Terminator (Applied Biosystems). Sequence reactions were analyzed on ABI-3730xl sequencer (Applied Biosystems) and assembled using Seqman II (DNAstar, Madison, WI, USA). Finally, a phylogenetic tree was built in MegAlign (DNAstar, Madison, WI, USA) and included the above mentioned reference sequences for assemblage A–E.

2.4. Questionnaire

A questionnaire was distributed among owners of the chinchillas to obtain additional information on each of the examined animals, including the housing (dimensions of the enclosures), hygiene (frequency of cleaning the enclosures, use of disinfectants and contact with other pet animals) and the participation in shows.

2.5. Statistical analysis

To examine putative risk factors for parasitism, generalized linear models (binomial error) were built (9.2, SAS Institute Inc., Cary, NC, USA). First, the factors 'origin' (pet owner/breeder [breeds animals for commercial purposes]), 'sex' (female/male), 'use of disinfectants' (yes/no), 'participating shows' (yes/no) and 'contact with other pets' (yes/no), and covariates 'age', 'stocking density' (number of

animals per m^3), and 'frequency of cleaning' (per month) were tested for unconditional associations with *Giardia* infection (presence/absence). The level of significance was set at $p < 0.20$. The importance of these variables was evaluated in a multivariate model by backward elimination using the likelihood ratio test of χ^2. To this end, the level of significance was set at $p < 0.05$. Finally, the probability of a *Giardia* infection for each of the observed values of the covariates and factors was calculated based on this model (The R Foundation for Statistical Computing, version 2.10.0).

3. Results

3.1. Ocurrence of Giardiaspp. and risk factor analysis

In 53 out of 80 animals (66.3%) *Giardia* cysts were found by microscopy. As illustrated in Fig. 1, there were only two variables that significantly contributed to the presence of *Giardia*, including age ($p < 0.005$) and participation in shows ($p < 0.05$). Younger animals were more infected with this parasite compared to older animals and participation in shows increased the risk of a *Giardia* infection.

3.2. Molecular characterization

The molecular characterization was performed on 22 samples, each representing one cage. The results of the direct sequencing and assemblage-specific approach at the level of assemblage and sub-assemblage are summarized in Table 1. In 21 samples amplification was obtained by one or both approaches. Direct sequencing of the bg gene solely yielded *G. duodenalis* assemblage B in 17 samples. In the remaining samples no amplification was found. The assemblage-specific PCRs revealed the presence of *G. duodenalis* in all samples, with the exception of sample H4. This approach confirmed the high prevalence of assemblage B (18/21), but also demonstrated the presence of assemblage C (15/21), A (11/21) and E (2/21). Mixed assemblage infections with at least two of these assemblages were observed

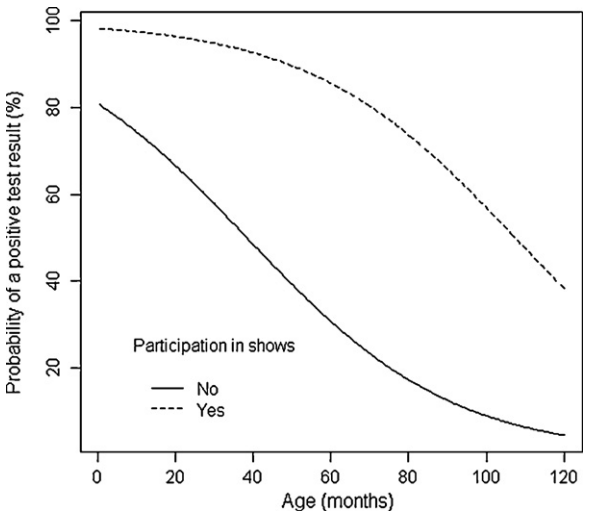

Fig. 1. The effect of age and participation in shows on the probability of a positive test result for *Giardia* in chinchillas.

Table 1
A comparison of direct sequencing targeting the β-giardin gene (bg) and assemblage specific PCR targeting triose phosphate isomerase gene (tpi-mixed) for the identification of 21 out of 22 *Giardia* samples from chinchillas at the level of assemblage and (sub-assemblage).

Sample	bg	tpi-mixed
A3	-	B(IV)
A7	B(IV)	A(II) + B(IV) + C
A11	B(IV)	A (II) + B + C
A14	B(III/IV)	B + C
A15	B(IV)	A(I) + B + C + E
A16	B(III/IV)	A(I/II) + B(IV)
A19	B(IV)	B(IV)
A21	B(IV)	B(IV-like) + C
A27	B(IV)	B(IV) + E
B2	B(IV)	A(II) + B(IV) + C
B3	B(IV)	B + C
C13	B(IV)	A + B + C
C15	B(IV)	B + C
E1	B(IV)	A + C
E5	B(IV)	B(IV)
F1	-	A + C
G1	-	A(I) + B(IV)
H2	B(IV)	B(IV) + C
H4	-	-
H11	-	C
H13	B(IV)	A(I/II) + B + C
H19	B(IV)	A + B + C

in 17 out of 21 samples. The specificity of the assemblage specific primers was confirmed by sequencing 32 PCR products (assemblage A: 7, assemblage B: 10, assemblage C: 14 and assemblage E: 1) and is illustrated in Fig. 2.

The substitution patterns defining the sub-assemblages for bg (assemblage B, Levecke et al., 2009) and tpi (assemblage A and B, Wielinga and Thompson, 2007) are summarized in Supplemental Table 1. For the bg gene, 15 out of the 17 assemblage B isolates were classified as sub-assemblage BIV based on the thymine at position 354. For the remaining two isolates (A14 and A16) a heterogonous template Y was found which indicated a mixed sub-assemblage BIII (cytosine) and BIV infection. For the tpi gene, sequence information was available for 10 assemblage B and 7 assemblage A isolates. All assemblage B isolates were grouped as sub-assemblage BIV, except for isolate A21 that belonged to sub-assemblage BIV-like. Five out of seven assemblage A isolates could be grouped in either sub-assemblage AI (A15, G1) or AII (A7, A11, B2). The remaining two isolates (A16, H13) were mixed sub-assemblage AI and AII infections.

4. Discussion

In the present study the occurrence of *Giardia* in pet chinchillas was examined, in combination with an analysis of the risk factors contributing to infection and of the role of these animals as a potential zoonotic reservoir using an assemblage specific approach. The results indicated that *Giardia* is highly prevalent in pet chinchillas (66.3%). The infection rate in the present study is higher than in previous studies where prevalence did not exceed 37% (Gurgel et al., 2005; Fialho et al., 2008). These differences are probably multi-factorial and may include discrepancies in the management of the animals and preservation of the sam-

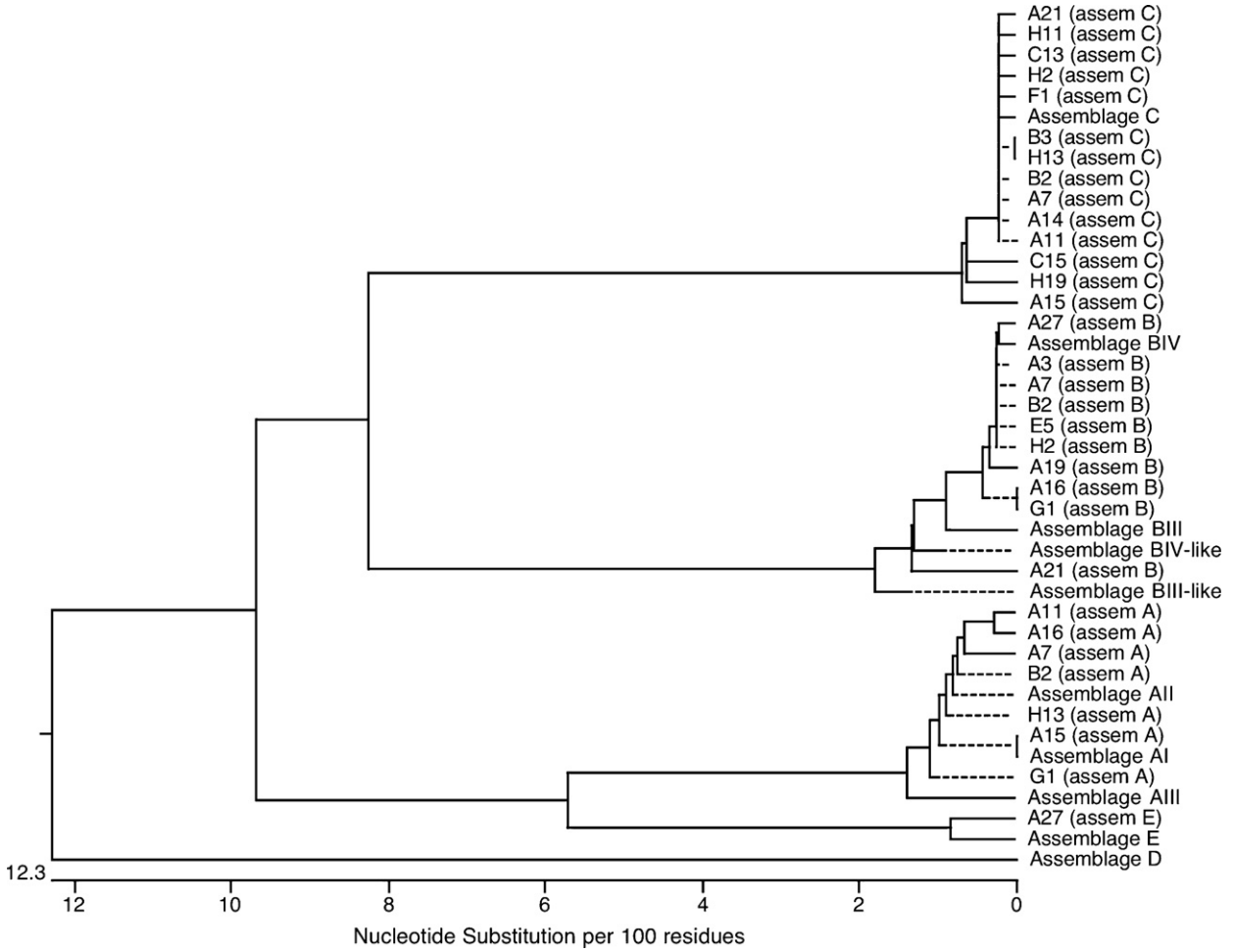

Fig. 2. The phylogenetic tree shown is generated form a nucleotide fragment (243 base pairs) of the triose phosphate isomerase of *Giardia duodenalis* assemblage A (7 sequences, Genbank™ accession numbers HQ397684–90), B (10 sequences, Genbank™ accession numbers HQ397691–95), C (15 sequences, Genbank™ accession numbers HQ397696–HQ397703) and E (1, sequence, Genbank™ accession number HQ397704) isolates found in chinchilla and reference sequences (Genbank™ accession number) for assemblage A (AI: L02120; AII: U57897) B (BIII: AY368163. BIII-like: AY368166; BIV-like: AY228635; BIV: AY368170), C (AF069563), D (DQ246216) and E (AY655705).

ples. In previous studies animals were individually housed, whereas the majority of animals in the present study were housed in groups of at least two animals. In addition, in a Brazilian study samples were maintained at −8 °C until further analysis (Gurgel et al., 2005) which may result in false negative test results due to disruption of the cysts (Olson et al., 1999). Youngsters and animals participating in shows were more at risk for a *Giardia* infection. The difference in age is not surprising and confirms the findings of surveys conducted in various host species (Claerebout et al., 2009; Geurden et al., 2010). The increased risk for animals attending shows can be explained by more frequent contact with other animals. However, chinchillas attending shows might also become more vulnerable for infections as a result of the stress associated with these shows (Stark and Barratt, 2009).

The molecular identification revealed that chinchillas harbor various assemblages of *G. duodenalis*, including assemblage A, B, C and E. All samples contained at least one of the assemblages A and B, which highlights the role of these animals as a potential reservoir for zoonotic transmission. The presence of assemblage C and E is unexpected, since these assemblages are assumed to be specific for canids and livestock, respectively. Cross-species transmission of *G. duodenalis* assemblage C (cats and humans) and E (dogs, cats, nonhuman primates and humans) has been reported in other host species (Sprong et al., 2009; Johnston et al., 2010). Yet, the frequency of cross-species transmission in these host species (<5%) is fractional compared to the occurrence of both assemblages in the chinchillas examined, particularly for assemblage C (71%). This discrepancy is probably due to the limitations of standard PCR protocols followed by sequencing, as this approach underestimates the occurrence of mixed assemblage infections. Therefore, the inclusion of assemblage-specific PCRs seems to be appropriate in future epidemiological surveys. The origin of these assemblages C and E in the chinchillas examined remains unclear, as these animals had not always contact with dogs or livestock, either directly or indirectly. The molecular analysis at the sub-assemblage level

revealed that assemblage A isolates were identified as AI and AII. Assemblage B isolates were grouped into BIII and BIV at the bg gene and into BIV and BIV-like at the tpi gene. At present, substitution patterns to determine sub-assemblages in analogy with the tpigene are not available for the bg gene. Recently an alternative substitution has been proposed, separating the sub-assemblage BIII (cytosine) from BIV (thymine) at position 354. However, its validity remains unknown (Levecke et al., 2009). The comparison of both genes revealed a good agreement in the assignment of isolates at the level of B sub-assemblage, underlining the potency of this novel classification.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetpar.2010.11.027.

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